Role of Angiotensin II in the Remodeling Induced by a Chronic Increase in Flow in Rat Mesenteric Resistance Arteries

Maud Cousin, Marc-Antoine Custaud, Céline Baron-Menguy, Bertrand Toutain, Odile Dumont, Anne-Laure Guihot, Emilie Vessières, Jean-François Subra, Daniel Henrion, Laurent Loufrani

Abstract—Angiotensin II is a potent growth factor involved in arterial wall homeostasis. In resistance arteries, chronic increases in blood flow induce a rise in diameter associated with arterial wall hypertrophy. Nevertheless, the role of angiotensin II in this remodeling is unknown. We investigated the effect of blocking angiotensin II production or receptor activation on flow-induced remodeling of mesenteric resistance arteries. Arteries were ligated in vivo to generate high-flow arteries compared with normal flow (control) vessels located at a distance. Arteries were isolated after 1 week for in vitro analysis. Arterial diameter, media surface, endothelial NO synthase expression, superoxide production, and extracellular signal–regulated kinase 1/2 phosphorylation were higher in high-flow than in control arteries. Angiotensin-converting enzyme inhibition (perindopril) and angiotensin II type 1 receptor blockade (candesartan) prevented arterial wall hypertrophy without affecting diameter enlargement. The nonselective vasodilator hydralazine had no effect on remodeling. Although perindopril and candesartan increased endothelial NO synthase expression in high-flow arteries, hypertrophy remained in rats treated with NO-nitro-L-arginine methyl ester and mice lacking endothelial NO synthase. Perindopril and candesartan reduced oxidative stress in high-flow arteries, but superoxide scavenging did not prevent hypertrophy. Both Tempol and the absence of endothelial NO synthase prevented the rise in diameter in high-flow vessels. Extracellular signal–regulated kinase 1/2 activation in high-flow arteries was prevented by perindopril and candesartan and not by hydralazine. Extracellular signal–regulated kinase 1/2 inhibition in vivo (U0126) prevented hypertrophy in high-flow arteries. Thus, a chronic rise in blood flow in resistance arteries induces a diameter enlargement involving NO and superoxide, whereas hypertrophy was associated with extracellular signal–regulated kinase 1/2 activation by angiotensin II. (Hypertension. 2010;55:109-115.)

Key Words: resistance arteries ■ blood flow ■ angiotensin II ■ ERK1/2 ■ shear stress ■ angiotensin I–converting enzyme inhibitors ■ angiotensin II type 1 receptor inhibitor

Angiotensin II is a potent vasoactive hormone involved in the regulation of vascular tone,1,2 in cell growth and apoptosis,3 and in cell migration and extracellular matrix deposition.4 Angiotensin II also has a central role in the functional and structural integrity of the vascular wall and in the pathogenesis of cardiovascular diseases.5–7

Chronic changes in blood flow occur in physiological conditions, such as exercise, pregnancy, or postnatal development, and in pathological conditions, such as arterial occlusive diseases, diabetes mellitus, or hypertension.1,8 A chronic increase in blood flow induces remodeling of the vascular wall to adapt arterial wall strain to the new hemodynamic conditions, as described in large blood vessels9 and in small resistance arteries.1,8 Indeed, in resistance arteries, a chronic increase in blood flow induces an increase in diameter associated with media thickening (hypertrophic remodeling).10–12 The mechanism involved in the rise in diameter is well described in large conductance arteries.9 In resistance arteries, less is known. We have shown previously that NO synthesis blockade or endothelial NO synthase (eNOS) knockout prevented the diameter enlargement without affecting hypertrophy.13 The mechanism involved in the hypertrophy because of a chronic rise in blood flow (shear stress) in resistance arteries remains unknown. The acute stimulation of mesenteric resistance arteries by flow (shear stress) induces a vasodilation, which is modulated by the local production of angiotensin II.14–16 In addition, angiotensin II has major trophic properties. Thus, we tested the hypothesis that angiotensin II could be involved in the hypertrophy after a chronic rise in blood flow in vivo. We used a model described...
previously in rats\textsuperscript{10,17} and mice,\textsuperscript{11,18} allowing for comparison of resistance arteries submitted chronically to high or normal blood flow in vivo without changes in systemic hemodynamic conditions or hormonal environment. In this model, blood flow is doubled compared with other mesenteric arteries.\textsuperscript{10,11,17} In this model, we tested the effect of angiotensin I–converting enzyme inhibition with perindopril and angiotensin II type 1 receptor blockade with candesartan on outward hypertrophic remodeling induced by high blood flow in a resistance artery in vivo. Hydralazine was used as a control “nonspecific” vasodilator treatment.\textsuperscript{19}

**Methods**

**Animals**

Twelve-week–old male Wistar rats (Ifla-Credo, Les Arbresles, France) were anesthetized (pentobarbital sodium, 50 mg/kg IP) and submitted to surgery to increase blood flow in a mesenteric resistance artery, as shown previously.\textsuperscript{11,13} Briefly, 2 first-order arteries were alternatively ligated near their bifurcation into second-order arteries. The nonligated middle artery was, thus, exposed to high flow (HF) (Figure 1A).

Rats were treated with perindopril (2 mg/kg per day; n = 12), candesartan (2 mg/kg per day; n = 12), hydralazine (200 mg/L per day in drinking water; n = 12), or tap water (control group; n = 12) for 1 week. Treatments were started 1 day before surgery.

In other series of experiments, 8 rats were treated with the superoxide dismutase mimetic Tempol (4-hydroxy-2,2,6,6-tetramethyl piperidinoxyl; 20 mg/kg per day), 8 other rats were treated with the extracellular signal–regulated kinase (ERK1/2) inhibitor U-0126 (1,4-diamino-2,3-dicyano-1,4-bis [2-aminophenylthio] butadiene; 30 mg/kg per day SC), and 6 rats were treated with N\textsuperscript{5},N\textsuperscript{6}-nitro-L-arginine methyl ester (l-NAME; 50 mg/kg per day, in drinking water) and hydralazine (300 mg/L per day in drinking water).

In another series of experiments, 3-month–old male mice lacking the gene encoding for eNOS (eNOS\textsuperscript{−/−}) and their control (eNOS\textsuperscript{+/+}) were used (n = 6 per group) and submitted to the protocol described above. One week after surgery, animals were anesthetized with sodium pentobarbital (50 mg/kg IP). The right femoral artery was cannulated for blood pressure measurement. Animals were then euthanized by CO\textsubscript{2} inhalation, the gut excised, and mesenteric arteries dissected, as described previously. HF and normal flow (NF) arteries were divided into 2 segments, proximal for the functional study and distal for histological and biochemical analysis.\textsuperscript{13}

The procedure followed in the care and euthanasia of the study animals was in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and was approved by the regional ethical committee (Ministère de l’Agriculture, France, authorization No. 49045).

**Histology**

A segment of each mesenteric artery was dissected, cannulated at one end, and ligated at the other end. The artery was then bathed in a 50 mg/L per day, in drinking water) and hydralazine (300 mg/L per day in drinking water).

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Western Blot Analysis of eNOS, p38, p42, and p44

As shown previously,\textsuperscript{23} arterial segments were homogenized, and proteins (20 µg of total protein from each sample) were separated by SDS-PAGE. After migration, proteins were transferred to polyvinylidene fluoride blotting membranes (Amersham). Membranes were then washed in Tris-buffered saline-Tween 20 buffer and blocked for 2 hours at room temperature. Membranes were incubated for 90 minutes at room temperature with the primary antibody (Transduction Laboratories; eNOS, 1/1000; p38, 1/500; phosphop38, 1/500; p42, 1/500; phospho-p42, 1/500; p44, 1/500; and phospho-p44, 1/500 in Tris-buffered saline-Tween 20), washed again (3 times for 10 minutes), and incubated with antirabbit/rabbit peroxidase-conjugated antibody (Amersham) in Tris-buffered saline-

![Figure 1. Schematic representation of the model used to study chronic changes in blood flow in mesenteric arteries in vivo (A). Arrows indicate location of the ligations of second-order arteries. The artery located between 2 ligated vessels was designated as an HF artery. Equivalent arteries located at distance were used as a control (NF). The images represent typical sections of NF and HF arteries 1 week after surgery. Internal arterial diameter (B) and media cross-sectional area (C) were measured under a pressure of 75 mm Hg. Arteries were isolated from rats treated with perindopril (PER), candesartan (TCV), hydralazine (HYD), or water (CONT, control; n = 12 rats per group). *P<0.05, HF vs NF. #P<0.05, TCV, PER, or HYD vs CONT.](image-url)
Diameter and Cross-Sectional Area

In NF arteries, ID (Figure 1B) was not significantly modified by perindopril, candesartan, or hydralazine. Mesenteric artery ID was significantly higher in HF than in NF arteries. The rise in diameter because of flow (HF arteries) was not affected by perindopril, candesartan, or hydralazine. Cross-sectional area (Figure 1C) was not significantly modified by perindopril, candesartan, or hydralazine in NF arteries. In HF arteries, cross-sectional area was significantly higher in control HF than in control NF arteries, showing that hypertrophic outward remodeling occurred in arteries submitted chronically to high blood flow (Figure 1C). On the other hand, in HF arteries, media cross-sectional area was significantly lower in perindopril- and candesartan-treated rats than in control HF arteries and was not different from NF arteries, suggesting that perindopril and candesartan prevented hypertrophy in HF arteries. In hydralazine-treated rats, HF artery cross-sectional area was significantly higher than in NF arteries but not significantly different from that in control HF arteries (Figure 1C).

eNOS Expression Level

In the 4 groups (control, hydralazine, perindopril, and candesartan), eNOS expression level was higher in HF arteries than in NF vessels (Figure 2A). In NF arteries, eNOS expression level was not significantly affected by perindopril, candesartan, or hydralazine. In HF arteries, eNOS expression level was significantly higher in perindopril-treated rats compared with control rats.

No significant diameter enlargement was observed in rats treated chronically with L-NAME plus hydralazine (Figure 2B), whereas in the same group, media cross-section was higher in HF than in NF arteries (Figure 2C). Similarly, no significant increase in diameter was observed in eNOS knockout mice, as shown previously. Nevertheless, in L-NAME plus hydralazine-treated rats and in eNOS knockout mice, media cross-section was higher in HF than in NF arteries (eNOS knockout mice: Figure S1 of the online Data Supplement; please see http://hyper.ahajournals.org).
Role of Reactive Oxygen Species in Remodeling

Superoxide production was higher in HF than in NF arteries in control rats, as well as in rats treated with perindopril, candesartan, or hydralazine (Figure 3A). In NF arteries, no significant labeling could be detected (Figure 3A). The increase in superoxide level was observed in the endothelium and in the tunica media (Figure S2).

Perindopril, candesartan, and hydralazine reduced superoxide production in HF arteries compared with HF arteries. In rats chronically treated with Tempol, superoxide production in HF arteries was equivalent to that in NF vessels (Figure 3A).

To test the role of superoxides in the hypertrophy occurring in HF arteries, rats were treated chronically with the superoxide dismutase mimetic Tempol. This treatment prevented the increase in diameter but not the rise in media cross-sectional area in HF arteries (Figure 3B and 3C).

The increased reactive oxygen species (ROS) production found in HF arteries was associated with an increased level of nitrotyrosine in the arterial wall, attenuated only by Tempol (Figure S3). The increased ROS level in HF arteries was also confirmed using rhodamine staining and electron paramagnetic resonance measurement of superoxide anions (Figures S4 and S5).

Mitogen-Activated Protein Kinase Activation

The ratio of phospho-ERK1/2:ERK1/2 (p42 and p44) was significantly higher in HF than in NF arteries (Figure 4A). On the other hand, the ratio of phospho-p38:p38 was not significantly affected by the chronic rise in flow (Figure 4B).

In rats treated with perindopril or candesartan, no rise in the ratio of phospho-ERK1/2 occurred in HF arteries (Figure 4A and 4B, right). Hydralazine did not prevent the rise in phospho-ERK1/2 in HF arteries.

Because perindopril and candesartan inhibited the activation of ERK1/2 by the chronic rise in blood flow, we tested
the effect of a chronic blockade of ERK1/2 on flow-mediated remodeling. The ERK1/2 inhibitor U-0126 was given to rats submitted to mesenteric artery ligation. Unlike the treatments with perindopril or candesartan (Figure 1), U-0126 did not prevent the rise in luminal diameter (Figure 5A). Nevertheless, in U-0126–treated rats, cross-sectional area was similar in HF and NF arteries (Figure 5B) suggesting that the treatment prevented flow-induced hypertrophy. The treatment with U-0126 did not significantly affect superoxide and nitrotyrosine levels in NF or HF arteries (Figures S2 and S3).

Discussion

Our study showed that angiotensin II was involved in the hypertrophy associated with the diameter enlargement of mesenteric resistance arteries submitted to a chronic increase in blood flow in vivo. Angiotensin II–dependent hypertrophy in arteries submitted to HF required ERK1/2 activation.

The model used allows for the study of similar resistance arteries submitted to HF or NF in vivo without changes in physiological hemodynamic conditions.10,11,13,17 HF–induced remodeling includes a diameter enlargement, an arterial wall hypertrophy, and an increased endothelium (NO)-dependent dilation.11,12,28 Although the increase in diameter occurring in response to a chronic rise in blood flow, hypertrophy, is usually associated with a higher risk of cardiovascular events.

Arterial wall hypertrophy associated with a reduction in external diameter is usual in hypertension, and hypertrophy associated with a diameter enlargement is common in diabetes mellitus and obesity.29,30 In these different pathological situations, reducing hypertrophy is usually presented as a positive evolution in response to a treatment.1

In agreement with previous studies,12,13,31 we found that eNOS expression level increased in HF arteries and that vasodilator treatments could not increase arterial diameter over that obtained in control rats. The effect of the treatments on eNOS expression could provide an explanation for the antitrophic effect of candesartan and perindopril. Nevertheless, hydralazine had a similar effect on eNOS expression level without preventing hypertrophy. In addition, our experiments performed in rats treated with L-NAME (in association with hydralazine to prevent hypertension) and eNOS knockout mice showed that the absence of the enzyme or its blockade did not prevent hypertrophy in HF arteries. Hydralazine, per se, induces outward remodeling equivalent to that obtained in high-flow arteries.19 Thus, overactivation of eNOS expression by candesartan and perindopril cannot explain the effect on hypertrophy. Similarly, an effect of lowering blood pressure (as seen with perindopril) cannot be involved in the loss of hypertrophy in the HF vessels, because candesartan has a similar effect as perindopril without affecting blood pressure. The effect of perindopril on blood pressure is progressive, as shown previously.13

The rise in ROS level found in HF arteries was visualized using dihydroethidine, 3-nitro-tyrosine (present study and our previous reports12,18), and rhodamine (data not shown). This excessive ROS production could be involved in the hypertrophy observed in HF arteries. Indeed, in hypertension, ROS have a key role in arterial wall hypertrophy, and angiotensin II is involved in this increased ROS production.32 Consistently, we found in the present study that perindopril and candesartan reduced the rise in ROS in HF arteries. Nevertheless, although ROS are involved in the diameter enlargement, as shown previously in the carotid13 and in the mesenteric artery,18 they were not involved in the hypertrophy because of the chronic rise in blood flow. Indeed, Tempol did not prevent the hypertrophy, although it suppressed the rise in ROS in HF arteries. We obtained a similar result with the NAD(P)H-oxidase inhibitor apocynin (data not shown and Belin De Chantemelle et al16). In addition, hydralazine also reduced the ROS level in HF arteries without preventing the hypertrophy. Although perindopril, candesartan, and hydralazine reduced ROS level in HF arteries, a significant difference in the ROS level remained between HF and NF arteries. Indeed, a rise in ROS level seems necessary for the diameter enlargement in association with NO, in agreement with previous studies.18,27,33 Nevertheless, our study does not exclude a possible role for hydrogen peroxide in hypertrophy, because Tempol reduces superoxide dismutase level, but it also induces a rise in hydrogen peroxide level as it acts as a superoxide dismutase mimetic. Indeed, hydrogen peroxide has been shown to play a role in angiotensin II–induced hypertrophy of the aortic wall.34

That hypertrophy occurred in both Tempol-treated rats and eNOS-deficient mice raises a question on the relation between flow and hypertrophy. It is admitted that a chronic increase in blood flow induces vasodilation and, consequently, circumferential wall stress. This latter is then the stimulus for the production of growth factors involved in smooth muscle cell proliferation and media hypertrophy.8 Our results suggest that flow (shear stress) may also have a role in the production of growth factors or at least in the stimulation of the local renin-angiotensin system. This is in agreement with our previous studies showing the involvement of the system in response to acute changes in flow in vitro4,18 or in vivo35 in the mesenteric vascular bed. Indeed, an early stimulation of the local production of angiotensin II could have a role in the inflammatory response necessary for the remodeling,36 because angiotensin II may stimulate the production of monocyte chemoattractant protein 1.37 Nevertheless, this issue remains to be further investigated.

Perindopril and candesartan prevented the increase in ERK1/2 phosphorylation found in HF arteries, but not hydralazine did not. This suggests that ERK1/2 activated by
Angiotensin II may be involved in the hypertrophy of HF arteries. Angiotensin II is essential for arterial wall hypertrophy in hypertension through the activation of ERK1/2. The most interesting novelty of the present study is that angiotensin II was involved in arterial wall hypertrophy because of a rise in flow (shear stress), a vasorelaxing stimulus, in conditions where blood pressure was normal. Indeed, angiotensin II has been shown to exert its trophic properties on the arterial wall independent of pressure. Our findings are also in agreement with our previous studies showing that acute stimulation of mesenteric resistance arteries by flow activates the local production of angiotensin II, which possesses trophic properties on the vascular wall, especially in resistance arteries, through the activation of ERK1/2, independent of blood pressure. As stated above, hydrogen peroxide might have a role in hypertrophy, because it has been shown to activate ERK1/2. Indeed, hypertrophy remains in high-flow arteries, although the rise in diameter was absent, and Tempol increases hydrogen peroxide production. Furthermore, we have shown previously that hydralazine could increase remodeling in old rats in association with overexpression of the superoxide dismutases involved in hydrogen peroxide production. Nevertheless, this issue remains to be further confirmed.

Perspectives

The findings of the present study might be of importance in pathological situations involving resistance artery remodeling. In many diseases, arterial remodeling occurs in association with changes in pressure and/or local blood flow. This is the case in hypertension, diabetes mellitus, or in ischemic diseases. Treatment of these diseases frequently involves angiotensin-converting enzyme inhibitors or angiotensin II type 1 receptor blockers, and their effect on the hypertrophy associated with flow-mediated remodeling of resistance arteries should be taken into consideration. For example, in hypertension associated with an excessive vasoconstrictor tone, diameter reduction and wall thickening are associated with constant wall stress, whereas, in essential hypertension, the rise in pressure and shear stress induces wall thickening without a change in diameter, and, consequently, wall stress increases. Hypertension because of angiotensin II has the same effect. Nevertheless, it is also important to consider that a rise in diameter without a concomitant hypertrophy might be deleterious and eventually lead to aneurism. To the same effect. Nevertheless, it is also important to consider that a rise in diameter without a concomitant hypertrophy might be deleterious and eventually lead to aneurism. To the best of our knowledge, our study is the first showing the direct involvement of angiotensin II in the remodeling induced solely by a chronic rise in blood flow or shear stress in resistance arteries. Flow-induced diameter increase is also involved in arterial occlusive diseases. Indeed, both angio genesis and flow-mediated enlargement of preexisting bypass arteries (arteriogenesis) are involved.

In conclusion, our findings showed that, in resistance arteries, the hypertrophy associated a chronic increase in blood flow involved angiotensin II and ERK1/2. These findings provide further evidence that angiotensin-converting enzyme inhibitors or angiotensin II type 1 receptor blockers might be attractive in ischemic diseases.

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Disclosures

None.

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Supplemental data:

Figure S1: Internal arterial diameter and media cross sectional area measured in mesenteric arteries submitted to high (HF) or normal blood flow (NF) in mice lacking eNOS (−/−) and in their control (+/+). Mean±SEM is shown (n=6 per group).

*p<0.05, HF versus NF

&*p<0.05, −/− compared to +/+
Figure S2. Reactive oxygen species (ROS) visualized using dihydroethidium staining in mesenteric resistance arteries submitted to a chronic increase in blood flow (HF) compared to control arteries exposed to normal flow (NF). ROS level was quantified by image analysis in rats treated with perindopril (PER), candesartan (TCV), hydralazine (HYD), U-0126 or water (control: CONT). The number of positive nuclei per 10,000µm² was quantified in the tunica media (A) and in the endothelium (B). Mean±SEM is shown (n=12 rats per group).

*p<0.05, HF versus NF
Figure S3. 3-nitrotyrosine (3-NT) expression level determined using Western-blot analysis of mesenteric resistance arteries submitted to a chronic increase in blood flow (HF) compared to control arteries exposed to normal flow (NF). 3-NT level was quantified and expressed as a ration to β-actin. Arteries were isolated from rats treated with perindopril (PER), candesartan (TCV), hydralazine (HYD), U-0126, tempol (TEMP) or water (control: CONT).

Method as described in the Material and Methods section with antibodies (anti-3-NT) from Transduction Laboratories (1/500).

Mean±SEM is shown (n=8 per group).

*p<0.05, HF versus NF

#p<0.05, treatment versus CONT

Figure S4. Reactive oxygen species (ROS) visualized using rhodamine staining (green) in mesenteric resistance arteries submitted to a chronic increase in blood flow (HF) compared to control arteries exposed to normal flow (NF). Nuclei were stained with DAPI (blue staining). ROS level was quantified by image analysis in rats treated with candesartan (TCV, panel B), tempol (Temp, panel C) or water (control: CONT, A). In negative control experiments rhodamine was omitted or tempol added acutely to the slide (D). A positive control was obtained with arteries isolated from LPS-treated rats. Fluorescence quantification is shown in the bargraph. Mean±SEM is presented (n=8 per group).

*P<0.05, HF versus NF
#P<0.05, TCV or Temp compared to CONT.
Figure S5: Electron Paramagnetic Resonance measurement of superoxide anions: HF and NF mesenteric arteries from 4 rats were pooled and incubated in a spin trap solution containing 500 mM 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidin (CMH, Noxygen), 25 mM deferoxamine (Sigma) and 5 mM N,N-diethyldithiocarbamate (DETC, Sigma) in a physiological salt solution at pH 7.4 and 37°C for 45 min. U46619 (stable thromboxane A2 mimetic, U46, 10 nM) was added during the spin trap incubation. Reaction was then stopped by freezing the sample in liquid nitrogen. Samples were then analyzed by EPR spectrometry. Spectra of the oxidized product of CMH (CMN) were recorded at 77°K using a flask Dewar. Acquisition parameters were as followed: Bo Field: 33416150 G, microwave power: 10 dB, amplitude modulation: 5 G, sweep time: 60 sec, gain: 300 and 3 scans. Signals were quantified by measuring the total amplitude, after correction of baseline and normalized to the protein quantity of the sample in mg/ml. Mean±SEM are presented (n=6 experiments per group with 5 rats per experiment). *P<0.05, CONT versus U46 #P<0.05, HF versus NF